

B20

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
23 January 2003 (23.01.2003)

PCT

(10) International Publication Number  
WO 03/006666 A2(51) International Patent Classification<sup>7</sup>: C12P 13/00

(21) International Application Number: PCT/EP02/06187

(22) International Filing Date: 6 June 2002 (06.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
101 33 667.5 11 July 2001 (11.07.2001) DE  
60/305,144 16 July 2001 (16.07.2001) US(71) Applicant (for all designated States except US): DE-  
GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düssel-  
dorf (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): SIEBELT, Nicole  
[DE/DE]; Am Sennebach 28, 33397 Rietberg (DE).  
WIDAWKA, Petra [DE/DE]; Lacerstrasse 12, 33615  
Bielefeld (DE). FARWICK, Mike [DE/DE]; Gus-  
tav-Adolf-Strasse 11, 33615 Bielefeld (DE).(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,  
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,  
VN, YU, ZA, ZM, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,  
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,  
NE, SN, TD, TG).

## Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv)) for US only

## Published:

— without international search report and to be republished  
upon receipt of that reportFor two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

WO 03/006666 A2

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE  
FAMILY(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the fol-  
lowing steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino  
acid, b) isolation of the desired L-amino acid from the fermentation broth, and c) isolation of the desired L-amino acid.  
The invention also relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the fol-  
lowing steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino  
acid, b) isolation of the desired L-amino acid from the fermentation broth, and c) isolation of the desired L-amino acid.

**Process for the Preparation of L-Amino Acids Using  
Strains of the Enterobacteriaceae Family**

**Field of the Invention**

This invention relates to a process for the preparation of  
5 L-amino acids, in particular L-threonine, using strains of  
the Enterobacteriaceae family in which at least one or more  
of the genes of the cysteine biosynthesis pathway (cysteine  
biosynthetic pathway) chosen from the group consisting of  
cysG, cysB, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD,  
10 cysN, cysC, cysJ, cysI, cysH, cysE and sbp is (are)  
enhanced.

**Prior Art**

L-Amino acids, in particular L-threonine, are used in human  
medicine and in the pharmaceuticals industry, in the  
15 foodstuffs industry and very particularly in animal  
nutrition.

It is known to prepare L-amino acids by fermentation of  
strains of Enterobacteriaceae, in particular *Escherichia*  
*coli* (*E. coli*) and *Serratia marcescens*. Because of their  
20 great importance, work is constantly being undertaken to  
improve the preparation processes. Improvements to the  
process can relate to fermentation measures, such as e.g.  
stirring and supply of oxygen, or the composition of the  
nutrient media, such as e.g. the sugar concentration during  
25 the fermentation, or the working up to the product form, by  
e.g. ion exchange chromatography, or the intrinsic output  
properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are  
used to improve the output properties of these  
30 microorganisms. Strains which are resistant to  
antimetabolites, such as e.g. the threonine analogue  $\alpha$ -  
amino- $\beta$ -hydroxyvaleric acid (AHV), or are auxotrophic for

acids, such as e.g. L-threonine, are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of  
5 strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

#### Object of the Invention

10 The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

#### Summary of the Invention

The invention provides a process for the fermentative  
15 preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which at least one or more of the nucleotide sequence(s) which code(s) for the genes of the cysteine biosynthesis pathway  
20 (cysteine biosynthetic pathway) chosen from the group consisting of cysG, cysB, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp is (are) enhanced.

The process according to the invention for the preparation  
25 of amino acids comprises the following steps:

- a) fermentation of the microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which one or more of the genes of the cysteine biosynthesis pathway chosen from  
30 the group consisting of cysG, cysB, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp, or nucleotide

sequences which code for them is/are enhanced, in particular over-expressed,

- b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
- 5 c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof optionally remaining in the product.

#### Detailed Description of the Invention

- 10 The use of endogenous genes is preferred. "Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences present in the population of a species.

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "enhancement" in this connection describes the increase in the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

- 30 By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is increased by at least 10%, 25%, 50%, 75%, 100%,

150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

5 The process comprises carrying out the following steps:

a) fermentation of microorganisms of the Enterobacteriaceae family in which one or more of the genes of the cysteine biosynthesis pathway chosen from the group consisting of cysG, cysB,  
10 cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp is (are) enhanced,

b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and  
15

c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

20 The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen  
25 from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.

30 Suitable strains, which produce L-threonine in particular, of the genus Escherichia, in particular of the species Escherichia coli, are, for example

- Escherichia coli TF427
- Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIGenetika MG442
- 5 Escherichia coli VNIIGenetika M1
- Escherichia coli VNIIGenetika 472T23
- Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132

- 10 Suitable L-threonine-producing strains of the genus *Serratia*, in particular of the species *Serratia marcescens*, are, for example

- Serratia marcescens* HNr21
- Serratia marcescens* TLR156
- 15 *Serratia marcescens* T2000

- Strains from the Enterobacteriaceae family which produce L-threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of:
- 20 resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic acid, resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate,
  - 25 resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a partial and compensable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine,
  - 30 resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine; resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine,

11

dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement  
 5 of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form,  
 10 enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

15 It has been found that microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after enhancement, in particular over-expression, of at least one or more of the genes of the cysteine biosynthesis pathway  
 20 chosen from the group consisting of cysG, cysB, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp.

The nucleotide sequences of the genes of *Escherichia coli* belong to the prior art (See following text references) and  
 25 can also be found in the genome sequence of *Escherichia coli* published by Blattner et al. (Science 277: 1453 - 1462 (1997)). The genes and activities of the cysteine biosynthesis pathway (cysteine biosynthetic pathway) are also described in summary form in Kredich (In: Neidhardt  
 30 (ed), *Escherichia coli* and *Salmonella*, American Society for Microbiology, Washington, D.C., USA: 514-527 (1996)).

cysG gene:

Description: Uroporphyrinogen III C-methyl-transferase;  
 precorrin-2 oxidase; ferrochelatase

11 Amino acid:

Uroporphyrinogen III C-methyl-transferase

- Reference: Peakman et al.; European Journal of Biochemistry 191(2): 315-323 (1990)  
Macdonald and Cole; Molecular and General Genetics 200(2): 328-334 (1985) Warren et al.; Biochemical Journal 265(3):725-729 (1990) Spencer et al.; FEBS Letters 335(1): 57-60 (1993)
- 5
- Accession No.: AE000412
- cysB gene:
- 10 Description: Positive regulator of the cys regulon, transcription activator
- Reference: Ostrowski et al.; Journal of Biological Chemistry 262(13): 5999-6005 (1987)  
Mascarenhas and Yudkin; Molecular and General Genetics 177(3): 535-539 (1980)  
Lochowska et al.; Journal of Biological Chemistry 276(3): 2098-2107 (2001)
- 15
- Accession No.: AE000225
- cysZ gene:
- 20 Description: Sulfate transporter
- Reference: Byrne et al.; Journal of Bacteriology 170(7): 3150-3157 (1988)
- Accession No.: AE000329
- cysK gene:
- 25 Description: Cysteine synthase A, O-acetylserine (thiol)-lyase A
- EC No.: 4.2.99.8
- Reference: Byrne et al.; Journal of Bacteriology 170(7): 3150-3157 (1988) Boronat et al.; Journal of General Microbiology 130: 673-685 (1984) Levy and Danchin; Molecular Microbiology 2(6): 777-783 (1988)
- 30
- Accession No.: AE000329
- Accession No.: AE000329



- cysM gene:  
Description: Cysteine synthase B, O-acetylserine  
(thiol)-lyase B  
EC No.: 4.2.99.8  
5 Reference: Sirko et al.; Journal of Bacteriology  
172(6): 3351-3357 (1990) Sirko et al.;  
Journal of General Microbiology 133: 2719-  
2725 (1987)  
Accession No.: AE000329
- 10 cysA gene:  
Description: ATP-binding protein of the sulfate  
transport system  
Reference: Sirko et al.; Journal of Bacteriology  
172(6): 3351-3357 (1990) Sirko et al.;  
15 Journal of General Microbiology 133: 2719-  
2725 (1987)  
Accession No.: AE000329
- cysW gene:  
Description: Membrane-bound sulfate transport protein  
20 Reference: Sirko et al.; Journal of Bacteriology  
172(6): 3351-3357 (1990)  
Accession No.: AE000329, AE000330
- cysU gene:  
Description: Permease protein of the sulfate transport  
25 system  
Reference: Sirko et al.; Journal of Bacteriology  
172(6): 3351-3357 (1990) Hryniewicz et al.;  
Journal of Bacteriology 172(6): 3358-3366  
(1990)  
30 Accession No.: AE000330  
Alternative gene name: cysT
- cysP gene:  
Description: Periplasmic thiosulfate-binding protein

- Reference: Hryniewicz et al.; Journal of Bacteriology  
172(6): 3358-3366 (1990) Sirko et al.;  
Journal of Bacteriology 177(14): 4134-4136  
(1995)
- 5 Accession No.: AE000330
- cysD gene:
- Description: Sub-unit 2 of ATP sulfurylase (ATP:sulfate  
adenylyl-transferase)
- EC No.: 2.7.7.4
- 10 Reference: Leyh et al.; Journal of Biological  
Chemistry 267(15): 10405-10410 (1992) Leyh  
et al.; Journal of Biological Chemistry  
263(5): 2409-2416 (1988)
- Accession No.: AE000358
- 15 cysN gene:
- Description: Sub-unit 1 of ATP sulfurylase (ATP:sulfate  
adenylyl-transferase)
- EC No.: 2.7.7.4
- Reference: Leyh et al.; Journal of Biological  
20 Chemistry 267(15): 10405-10410 (1992) Leyh  
et al.; Journal of Biological Chemistry  
263(5): 2409-2416 (1988) Leyh and Suo;  
Journal of Biological Chemistry 267(1):  
542-545 (1992)
- 25 Accession No.: AE000358
- cysC gene:
- Description: Adenylyl sulfate kinase (APS kinase)
- EC No.: 2.7.1.25
- Reference: Leyh et al.; Journal of Biological  
30 Chemistry 267(15): 10405-10410 (1992) Leyh  
et al.; Journal of Biological Chemistry  
263(5): 2409-2416 (1988)
- Accession No.: AE000358

## cysJ gene:

Description: Flavoprotein of NADPH sulfite reductase  
EC No.: 1.8.1.2  
Reference: Ostrowski et al.; Journal of Biological  
5 Chemistry 264(27): 15796-15808 (1989) Li et  
al.; Gene 53(2-3): 227-234 (1987) Gaudu and  
Fontecave; European Journal of Biochemistry  
226(2): 459-463 (1994) Eschenbrenner et  
10 al.; Journal of Biological Chemistry  
270(35): 20550-20555 (1995)  
Accession No.: AE000360  
Alternative gene name: cysP

## cysI gene:

Description: Haemoprotein of NADPH sulfite reductase  
15 EC No.: 1.8.1.2  
Reference: Ostrowski et al.; Journal of Biological  
Chemistry 264(26): 15726-15737 (1989) Li et  
al.; Gene 53(2-3): 227-234 (1987) Gaudu and  
Fontecave; European Journal of Biochemistry  
20 226(2): 459-463 (1994)  
Accession No.: AE000360  
Alternative gene name: cysQ

## cysH gene:

Description: Phosphoadenosine phosphosulfate reductase  
25 (PAPS reductase)  
EC No.: 1.8.99.4  
Reference: Ostrowski et al.; Journal of Biological  
Chemistry 264(26): 15726-15737 (1989) Krone  
et al.; Molecular and General Genetics  
30 225(2): 314-319 (1991) Li et al.; Gene  
53(2-3): 227-234 (1987) Berendt et al.;  
European Journal of Biochemistry 233(1):  
347-356 (1995)  
Accession No.: AE000360

## cysE gene:

Description: Serine acetyl-transferase

EC No.: 2.3.1.30

Reference: Denk and Böck; Journal of General  
5 Microbiology 133, 515-25 (1987)

Accession No.: AE000438

## sbp gene:

Description: Periplasmic sulfate-binding protein

Reference: Hellings and Evans, European Journal of  
10 Biochemistry 149(2): 363-373 (1985) Sirko  
et al.; Journal of Bacteriology 177(14):  
4134-4136 (1995) Jacobson et al.; Journal  
of Biological Chemistry 266(8): 5220-5225  
(1991)

15 Accession No.: AE000466

The nucleic acid sequences can be found in the databanks of  
the National Center for Biotechnology Information (NCBI) of  
the National Library of Medicine (Bethesda, MD, USA), the  
nucleotide sequence databank of the European Molecular  
20 Biologies Laboratories (EMBL, Heidelberg, Germany or  
Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima,  
Japan).

The genes described in the text references mentioned can be  
used according to the invention. Alleles of the genes which  
25 result from the degeneracy of the genetic code or due to  
"sense mutations" of neutral function can furthermore be  
used.

To achieve an enhancement, for example, expression of the  
genes or the catalytic properties of the proteins can be  
30 increased. The two measures can optionally be combined.

To achieve an over-expression, the number of copies of the  
corresponding genes can be increased, or the promoter and  
regulation region or the ribosome binding site upstream of

the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-threonine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80: 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169 (1989)), in Hamilton (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)), pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Sciences USA 80 (21): 6557-6561 (1983)) can be used. A strain transformed with a plasmid vector, where the plasmid vector carries at least one or more of the genes

chosen from the group consisting of cysG, cysB, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp or nucleotide sequences which code for these, can be employed in a process according to the  
5 invention.

It is also possible to transfer mutations which affect the expression of the particular gene into various strains by sequence exchange (Hamilton et al. (Journal of Bacteriology 171: 4617 - 4622 (1989))), conjugation or transduction.

- 10 It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of  
15 reduced nicotinamide adenine dinucleotide phosphate or enzymes of glycolysis or PTS enzymes, in addition to enhancement of one or more of the genes of the cysteine biosynthesis pathway chosen from the group consisting of cysG, cysB, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD,  
20 cysN, cysC, cysJ, cysI, cysH, cysE and sbp.

Thus, for example, at the same time one or more of the genes chosen from the group consisting of

- the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and  
25 threonine synthase (US-A-4,278,765),
- the pyc gene of Corynebacterium glutamicum which codes for pyruvate carboxylase (WO 99/18228),
- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231: 332-336  
30 (1992)),
- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),

- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- 5 • the mqo gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1 013 765),
- the thrE gene of Corynebacterium glutamicum which codes  
10 for threonine export (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983))
- 15 • the hns gene which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212: 199-202 (1988)),
- the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994)),
- 20 • the fba gene which codes for fructose biphosphate aldolase (Biochemical Journal 257: 529-534 (1989)),
- the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- 25 • the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase

system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),

- the ptsG gene which codes for the glucose-specific IIBC component (Journal of Biological Chemistry 261: 16398-16403 (1986)),
  - the lrp gene which codes for the regulator of the leucine regulon (Journal of Biological Chemistry 266: 10768-10774 (1991)),
  - the mopB gene which codes for 10 Kd chaperone (Journal of Biological Chemistry 261: 12414-12419 (1986)) and is also known by the name groES,
  - the ahpC gene of the ahpCF operon which codes for the small sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92: 7617-7621 (1995))
  - the ahpF gene of the ahpCF operon which codes for the large sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92: 7617-7621 (1995))
- can be enhanced, in particular over-expressed.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to enhancement of one or more of the genes chosen from the group consisting of cysG, cysB, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp, for one or more of the genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Ravnikar and Somerville (Journal of Bacteriology 169: 4716-4721 (1987))),



- the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Vogel et al. (Archives in Microbiology 149: 36-42 (1987))),
- the gene product of the open reading frame (orf) yjfa  
5 (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- 10 • the pckA gene which codes for the enzyme phosphoenolpyruvate carboxykinase (Medina et al. (Journal of Bacteriology 172: 7151-7156 (1990))),
- the poxB gene which codes for pyruvate oxidase (Grabau and Cronan (Nucleic Acids Research 14 (13), 5449-5460  
15 (1986))),
- the aceA gene which codes for the enzyme isocitrate lyase (Matsuoko and McFadden (Journal of Bacteriology 170, 4528-4536 (1988))),
- the dgsA gene which codes for the DgsA regulator of the  
20 phosphotransferase system (Hosono et al. (Bioscience, Biotechnology and Biochemistry 59: 256-251 (1995)) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor (Jahreis et al. (Molecular and General Genetics 226:  
25 332-336 (1991)) and is also known by the name of the cra gene, and
- the rpoS gene which codes for the sigma<sup>38</sup> factor (WO 01/05939) and is also known under the name of the katF gene,
- 30 to be attenuated, in particular eliminated or for the

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme or protein with a low activity or inactivates the corresponding enzyme or protein or gene, and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to enhancement of one or more of the genes of the cysteine biosynthesis pathway chosen from the group consisting of cysG, cysB, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General  
5 Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower  
10 oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as  
15 a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium  
20 phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-  
25 containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be  
30 employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

- Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.
- 5 Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or
- 10 oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually
- 15 reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)), or it can take place by

20 reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine,

25 L-homoserine and L-lysine, in particular L-threonine.

The minimal (M9) and complete media (LB) for Escherichia coli used are described by J.H. Miller (A short course in bacterial genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli

30 and all techniques of restriction, ligation, Klenow and alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of Escherichia coli

35 is carried out by the method of Dooly et al. (Microbiology

of the National Academy of Sciences of the United States of America (1989) 86: 2172-2175).

The incubation temperature for the preparation of strains and transformants is 37°C.

## 5 Example 1

Preparation of L-threonine using the cysB gene

1a) Construction of the expression plasmid pTrc99AcysB

The cysB gene from E. coli K12 is amplified using the polymerase chain reaction (PCR) and synthetic  
10 oligonucleotides. Starting from the nucleotide sequence of the cysB gene in E. coli K12 MG1655 (Accession Number AE000225, Blattner et al. (Science 277: 1453-1462 (1997))), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

15 cysB1: 5' - GCGTCTAAGTGGATGGTTTAAC - 3' (SEQ ID No. 1)

cysB2: 5' - GGTGCCGAAAATAACGCAAG - 3' (SEQ ID No. 2)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany).  
20 A DNA fragment approx. 1000 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is  
25 ligated according to the manufacturer's instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10.

Selection of plasmid-carrying cells takes place on LB agar,  
30 to which 50 µg/ml kanamycin are added. After isolation of

cleaved with the restriction enzymes HindIII and XbaI and, after separation in 0.8% agarose gel, the cysB fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligation is carried out with the cysB fragment isolated. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes ScaI and SmaI. The plasmid is called pTrc99AcysB (Figure 1).

1b) Preparation of L-threonine with the strain  
MG442/pTrc99AcysB

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99AcysB described in example Ia and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 µg/ml ampicillin. The strains MG442/pTrc99AcysB and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm

on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 5 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. For complete induction of the expression of the *cysB* gene, 100 mg/l isopropyl β-D-thiogalactopyranoside (IPTG) are added in 10 parallel batches. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer 15 from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) 20 by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	Additives	OD (660 nm)	L-Threonine g/l
MG442	-	5.6	1.4
MG442/pTrc99A	-	3.8	1.3
MG442/pTrc99AcysB	-	4.4	1.7
MG442/pTrc99AcysB	IPTG	5.4	2.0

Example 2

Preparation of L-threonine using the cysK gene

2a) Construction of the expression plasmid pTrc99AcysK

The cysK gene from E. coli K12 is amplified using the  
5 polymerase chain reaction (PCR) and synthetic  
oligonucleotides. Starting from the nucleotide sequence of  
the cysK gene in E. coli K12 MG1655 (Accession Number  
AE000329, Blattner et al. (Science 277: 1453-1462 (1997))),  
PCR primers are synthesized (MWG Biotech, Ebersberg,  
10 Germany):

cysK1: 5' - CAGTTAAGGACAGGCCATGAG - 3' (SEQ ID No. 3)

cysK2: 5' - GCTGGCATTACTGTTGCAATTC - 3' (SEQ ID No. 4)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR  
is isolated according to the manufacturer's instructions  
15 with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany).  
A DNA fragment approx. 1000 bp in size can be amplified  
with the specific primers under standard PCR conditions  
(Innis et al. (1990) PCR Protocols. A Guide to Methods and  
Applications, Academic Press) with Pfu-DNA polymerase  
20 (Promega Corporation, Madison, USA). The PCR product is  
ligated according to the manufacturer's instructions with  
the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning  
Kit, Invitrogen, Groningen, The Netherlands) and  
transformed into the E. coli strain TOP10.

25 Selection of plasmid-carrying cells takes place on LB agar,  
to which 50 µg/ml kanamycin are added. After isolation of  
the plasmid DNA, the vector pCR-Blunt II-TOPO-cysK is  
cleaved with the restriction enzymes SpeI and XbaI and,  
after separation in 0.8% agarose gel, the cysK fragment is  
30 isolated with the aid of the QIAquick Gel Extraction Kit  
(QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia  
Lundbeck, Eppelheim, Germany) is cleaved with the enzyme XbaI



and ligation is carried out with the cysK fragment isolated. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which  
 5 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes HindIII and PvuII. The plasmid is called pTrc99AcysK (Figure 2).

2b) Preparation of L-threonine with the strain  
 10 MG442/pTrc99AcysK

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

15 The strain MG442 is transformed with the expression plasmid pTrc99AcysK described in example 2a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 µg/ml ampicillin. The strains MG442/pTrc99AcysK and MG442/pTrc99A are formed in this manner. Selected  
 20 individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained  
 25 in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm  
 30 on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin).

30 g/l CaCO<sub>3</sub>, 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 2.

15

Table 2

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AcysK	5.6	2.1

### Example 3

Preparation of L-threonine using the cysM gene

3a) Construction of the expression plasmid pTrc99AcysM

20 The cysM gene from E. coli K12 is amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysM gene in E. coli K12 MG1655 (Accession Number

AE000329, Blattner et al. (Science 277: 1453-1462 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The sequences of the primers are modified such that recognition sites for restriction enzymes are formed.

- 5 The recognition sequence for XbaI is chosen for the cysM1 primer and the recognition sequence for HindIII for the cysM2 primer, which are marked by underlining in the nucleotide sequence shown below:

cysM1: 5' - CGCATCAGTCTAGACCACGTTAGGATAG - 3'  
10 (SEQ ID No. 5)

cysM2: 5' - CATCAGTCTCCGAAGCTTTTAATCC - 3'  
(SEQ ID No. 6)

- The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions  
15 with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 950 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase  
20 (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer's instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10.

- 25 Selection of plasmid-carrying cells takes place on LB agar, to which 50 µg/ml kanamycin are added. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-cysM is cleaved with the restriction enzymes HindIII and XbaI and, after separation in 0.8% agarose gel, the cysM fragment is  
30 isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligation is carried out with the cysM

fragment. The ligation mixture is transformed into E. coli strain TOP10.

(Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes EcoRV, Eco91I, PstI and SspI. The plasmid is called pTrc99AcysM (Figure 3).

3b) Preparation of L-threonine with the strain  
MG442/pTrc99AcysM

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99AcysM described in example 3a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 µg/ml ampicillin. The strains MG442/pTrc99AcysM and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of

L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 3.

Table 3

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AcysM	1.6	2.0

#### 15 Example 4

Preparation of L-threonine using the cysP, cysU, cysW and cysA genes

##### 4a) Construction of the expression plasmid pTrc99AcysPUWA

The cysP, cysU, cysW and cysA genes from E. coli K12 are amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysP, cysU, cysW and cysA genes in E. coli K12 MG1655 (Accession Number AE000329 and AE000330,

Blattner et al. (Science 277: 1453-1462 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The sequences of the primers are modified such that recognition sites for restriction enzymes are formed. The  
5 recognition sequence for XbaI is chosen for the cysPUWA1 primer and the recognition sequence for HindIII for the cysPUWA2 primer, which are marked by underlining in the nucleotide sequence shown below:

10 cysPUWA1: 5' - GTCTCTAGATAAATAAGGGTGC GCAATGGC - 3'  
(SEQ ID No. 7)

cysPUWA2: 5' - CCGGGCGTTTAAAGCTTCACTCAACC - 3'  
(SEQ ID No. 8)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions  
15 with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 3900 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase  
20 (Promega Corporation, Madison, USA). The PCR product is cleaved with the restriction enzymes XbaI and HindIII and ligated with the vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden), which has been digested with the enzymes XbaI and HindIII. The E. coli strain XL1-Blue MRF'  
25 (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes BamHI, EcoRV, MluI, NdeI  
30 and SspI. The plasmid is called pTrc99AcysPUWA (Figure 4).

4b) Preparation of L-threonine with the strain  
MG442/pTrc99AcysPUWA

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited  
5 as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99AcysPUWA described in example 4a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar  
10 with 50 µg/ml ampicillin. The strains MG442/pTrc99AcysPUWA and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l  
15 glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  
20  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250 µl portions of this preculture are transinoculated into  
25 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. For complete induction of the expression of the cysPUWA genes, 100 mg/l  
30 isopropyl β-D-thiogalactopyranoside (IPTG) are added in parallel batches. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the  
35 culture suspension is determined with an LP2W photometer

from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 4.

Table 4

Strain	Additives	OD (660 nm)	L-Threonine g/l
MG442	-	5.6	1.4
MG442/pTrc99A	-	3.8	1.3
MG442/pTrc99AcysPUWA	-	5.5	1.7
MG442/pTrc99AcysPUWA	IPTG	6.5	2.1

10

#### Example 5

Preparation of L-threonine using the cysD, cysN and cysC genes

5a) Construction of the expression plasmid pTrc99AcysDNC

15 The cysD, cysN and cysC genes from E. coli K12 are amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysD, cysN and cysC genes in E. coli K12 MG1655 (Accession Number AE000358, Blattner et al. (Science 267: 1453-1462 (1997))), PCR primers are synthesized (MWG



are modified such that recognition sites for restriction enzymes are formed. The recognition sequence for XbaI is chosen for the cysDNC1 primer and the recognition sequence for HindIII for the cysDNC2 primer, which are marked by underlining in the nucleotide sequence shown below:

cysDNC1: 5' - GCAAGAAAATAGCGGTCTAGATAAGGAACG - 3'  
(SEQ ID No. 9)

cysDNC2: 5' - CATGGAAAGCTTGTGGTGTCTCAGG - 3'  
(SEQ ID No. 10)

10 The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 3000 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is cleaved with the restriction enzymes XbaI and HindIII and ligated with the vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden), which has been digested with the enzymes XbaI and HindIII. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes EcoRV, HincII, NruI, PvuI and ScaI. The plasmid is called pTrc99AcysDNC (Figure 5).

5b) Preparation of L-threonine with the strain MG442/pTrc99AcysDNC

30 The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99AcysDNC described in example 5a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 µg/ml ampicillin. The strains MG442/pTrc99AcysDNC  
5 and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of  
10 L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated  
15 and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ ,  
20 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the  
25 medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined  
30 in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 5.

Table 5

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AcysDNC	5.1	2.5

Example 6

Preparation of L-threonine using the *cysJ* and *cysI* genes

5 6a) Construction of the expression plasmid pTrc99AcysJI

The *cysJ* and *cysI* genes from *E. coli* K12 are amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the *cysJ* and *cysI* genes in *E. coli* K12 MG1655 (Accession  
10 Number AE000360, Blattner et al. (Science 277: 1453-1462 (1997))), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

*cysJI1*: 5' - CTGGAACATAACGACGCATGAC - 3' (SEQ ID No. 11)

*cysJI2*: 5' - GACCGGGCTGATGGTTAATCC - 3' (SEQ ID No. 12)

15 The chromosomal *E. coli* K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 3550 bp in size can be amplified with the specific primers under standard PCR conditions  
20 (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer's instructions with

the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10.

Selection of plasmid-carrying cells takes place on LB agar, to which 50 µg/ml kanamycin are added. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-cysJI is cleaved with the restriction enzymes HindIII and XbaI and, after separation in 0.8% agarose gel, the cysJI fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligation is carried out with the cysJI fragment isolated. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes AccI, ClaI and SphI. The plasmid is called pTrc99AcysJI (Figure 6).

6b) Preparation of L-threonine with the strain  
MG442/pTrc99AcysJI

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99AcysJI described in example 6a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 µg/ml ampicillin. The strains MG442/pTrc99AcysJI and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose, 20 g/l agar, 50 µg/ml ampicillin. The formation of

L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 6.

Table 6

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AcysJI	6.3	2.5

Example 7

Preparation of L-threonine using the cysH gene

5 7a) Construction of the expression plasmid pTrc99AcysH

The cysH gene from E. coli K12 is amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysH gene in E. coli K12 MG1655 (Accession Number  
10 AE000360, Blattner et al. (Science 277: 1453-1462 (1997))), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

cysH1: 5' - GGCAAACAGTGAGGAATCTATG - 3' (SEQ ID No. 13)

cysH2: 5' - GTCCGGCAATATTTACCCTTC - 3' (SEQ ID No. 14)

15 The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 800 bp in size can be amplified with the specific primers under standard PCR conditions (Innis  
20 et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer's instructions with

the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10.

Selection of plasmid-carrying cells takes place on LB agar, to which 50 µg/ml kanamycin are added. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-cysH is cleaved with the restriction enzymes HindIII and XbaI and, after separation in 0.8% agarose gel, the cysH fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligation is carried out with the cysH fragment isolated. The E. coli strain XL1-Blue MRF<sup>+</sup> (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes HincII and MluI. The plasmid is called pTrc99AcysH (Figure 7).

7b) Preparation of L-threonine with the strain MG442/pTrc99AcysH

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99AcysH described in example 7a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 µg/ml ampicillin. The strains MG442/pTrc99AcysH and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l Na<sub>2</sub>HPO<sub>4</sub>\*2H<sub>2</sub>O, 1.5 g/l KH<sub>2</sub>PO<sub>4</sub>, 1 g/l NH<sub>4</sub>Cl, 0.1 g/l MgSO<sub>4</sub>\*7H<sub>2</sub>O, 2 g/l glucose, 50 g/l agar, 10 µg/l ampicillin. The formation of

L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 7.



Table 7

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AcysH	4.1	2.7

## Brief Description of the Figures:

Figure 1: Map of the plasmid pTrc99AcysB containing the  
5 cysB gene.

Figure 2: Map of the plasmid pTrc99AcysK containing the  
cysK gene.

Figure 3: Map of the plasmid pTrc99AcysM containing the  
cysM gene.

10 Figure 4: Map of the plasmid pTrc99AcysPUWA containing  
the cysP, cysU, cysW and cysA genes.

Figure 5: Map of the plasmid pTrc99AcysDNC containing the  
cysD, cysN and cysC genes.

15 Figure 6: Map of the plasmid pTrc99AcysJI containing the  
cysJ and cysI genes.

Figure 7: Map of the plasmid pTrc99AcysH containing the  
cysH gene.

The length data are to be understood as approx. data. The  
abbreviations and designations used have the following  
20 meaning:-

\* Amp: Ampicillin resistance gene

- lacI: Gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- cysB: Coding region of the cysB gene
- 5 • cysK: Coding region of the cysK gene
- cysM: Coding region of the cysM gene
- cysP: Coding region of the cysP gene
- cysU: Coding region of the cysU gene
- cysW: Coding region of the cysW gene
- 10 • cysA: Coding region of the cysA gene
- cysD: Coding region of the cysD gene
- cysN: Coding region of the cysN gene
- cysC: Coding region of the cysC gene
- cysJ: Coding region of the cysJ gene
- 15 • cysI: Coding region of the cysI gene
- cysH: Coding region of the cysH gene
- 5S: 5S rRNA region
- rrnBT: rRNA terminator region

The abbreviations for the restriction enzymes have the  
 20 following meaning .

- AccI: Restriction endonuclease from *Acinetobacter calcoaceticus*
- BamHI: Restriction endonuclease from *Bacillus amyloliquefaciens*

- BstEII: Restriction endonuclease from *Bacillus stearothermophilus* ATCC 12980
- ClaI: Restriction endonuclease from *Caryophannon latum*
- EcoRI: Restriction endonuclease from *Escherichia coli*  
5 RY13
- EcoRV: Restriction endonuclease from *Escherichia coli*  
B946
- HincII: Restriction endonuclease from *Haemophilus influenzae* R<sub>c</sub>
- 10 • HindIII: Restriction endonuclease from *Haemophilus influenzae*
- MluI: Restriction endonuclease from *Micrococcus luteus*  
IFO 12992
- NdeI: Restriction endonuclease from *Neisseria*  
15 *dentrificans*
- NruI: Restriction endonuclease from *Norcadia ruba* (ATCC  
15906)
- PauI: Restriction endonuclease from *Paracoccus*  
*alcaliphilus*
- 20 • PvuI: Restriction endonuclease from *Proteus vulgaris*  
(ATCC 13315)
- PvuII: Restriction endonuclease from *Proteus vulgaris*  
(ATCC 13315)
- ScaI: Restriction endonuclease from *Streptomyces*  
25 *caespitosus*
- SmaI: Restriction endonuclease from *Serratia marcescens*

- SpeI: Restriction endonuclease from *Sphaerotilus* species  
ATCC 13923
- SphI: Restriction endonuclease from *Streptomyces*  
*phaeochromogenes*
- 5 • SspI: Restriction endonuclease from *Sphaerotilus* species  
ATCC 13925
- XbaI: Restriction endonuclease from *Xanthomonas*  
*campestris*

**What is claimed is:**

1. A process for the preparation of L-amino acids, in particular L-threonine, which comprises carrying out the following steps:
  - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which at least one or more of the genes of the cysteine biosynthesis pathway chosen from the group consisting of cysG, cysB,  
10 cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp, or nucleotide sequences which code for these, is (are) enhanced, in particular over-expressed,
  - 15 b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof optionally remaining in the product.
- 20 2. A process as claimed in claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.
- 25 3. A process as claimed in claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 30 4. A process as claimed in claim 1, wherein the expression of the polynucleotide (s) which code(s) for one or more of the genes of cysteine biosynthesis chosen from the group consisting of cysG, cysB, cysZ, cysK, cysM, cysA,

cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp is increased.

5. A process as claimed in claim 1, wherein the regulatory and/or catalytic properties of the polypeptides (proteins) for which the polynucleotides cysG, cysB, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp code are improved or increased.
6. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
  - 6.2 the pyc gene which codes for pyruvate carboxylase,
  - 6.3 the pps gene which codes for phosphoenol pyruvate synthase,
  - 6.4 the ppc gene which codes for phosphoenol pyruvate carboxylase,
  - 6.5 the pntA and pntB genes which code for transhydrogenase,
  - 6.6 the rhtB gene which imparts homoserine resistance,
  - 6.7 the mgo gene which codes for malate:quinone oxidoreductase,
  - 6.8 the rhtC gene which imparts threonine resistance.

- 6.9 the thrE gene which codes for the threonine export protein
- 6.10 the gdhA gene which codes for glutamate dehydrogenase
- 5 6.11 the hns gene which codes for the DNA-binding protein HLP-II,
- 6.12 the pgm gene which codes for phosphoglucomutase,
- 10 6.13 the fba gene which codes for fructose biphosphate aldolase,
- 6.14 the ptsH gene which codes for the phosphohistidine protein hexose phosphotransferase,
- 15 6.15 the ptsI gene which codes for enzyme I of the phosphotransferase system,
- 6.16 the crr gene which codes for the glucose-specific IIA component,
- 6.17 the ptsG gene which codes for the glucose-specific IIBC component,
- 20 6.18 the lrp gene which codes for the regulator of the leucine regulon,
- 6.19 the mopB gene which codes for 10 Kd chaperone,
- 6.20 the ahpC gene which codes for the small sub-unit of alkyl hydroperoxide reductase,
- 25 6.21 the ahpF gene which codes for the large sub-unit of alkyl hydroperoxide reductase,

is or are enhanced, in particular over-expressed, are

7. A process as claimed in claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:
- 7.1 the tdh gene which codes for threonine dehydrogenase,
  - 7.2 the mdh gene which codes for malate dehydrogenase,
  - 10 7.3 the gene product of the open reading frame (orf) yjfa,
  - 7.4 the gene product of the open reading frame (orf) yjfp,
  - 15 7.5 the pckA gene which codes for phosphoenolpyruvate carboxykinase,
  - 7.6 the poxB gene which codes for pyruvate oxidase,
  - 7.7 the aceA gene which codes for isocitrate lyase,
  - 7.8 the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,
  - 20 7.9 the fruR gene which codes for the fructose repressor,
  - 7.10 the rpoS gene which codes for the sigma<sup>38</sup> factor

25 is or are attenuated, in particular eliminated or reduced in expression, are fermented.



Figure 1:

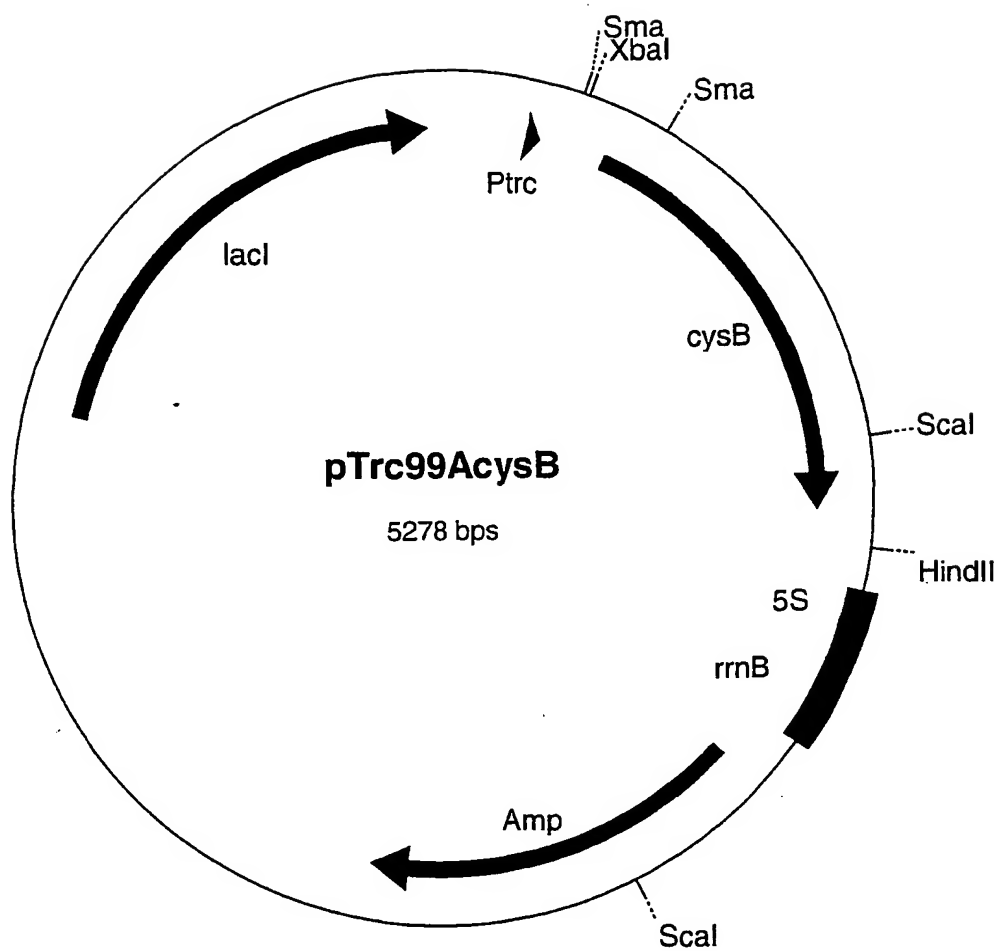


Figure 2:

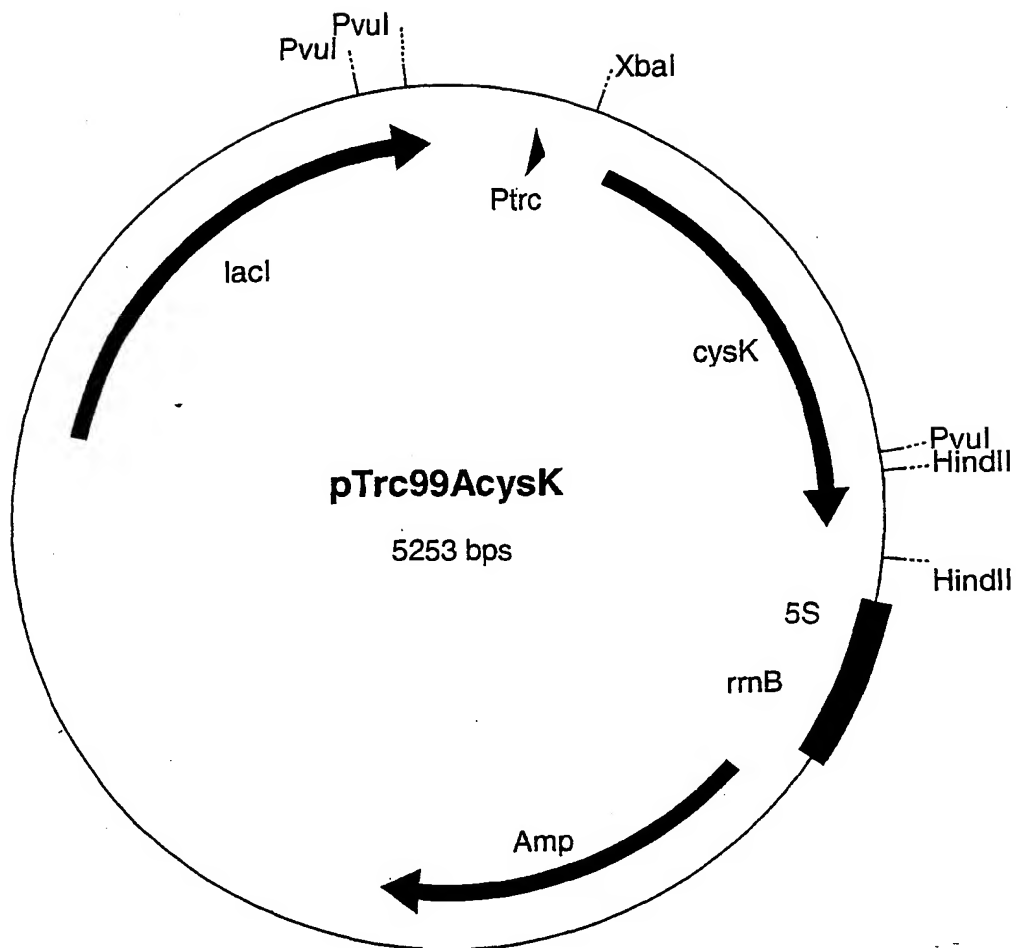


Figure 3:

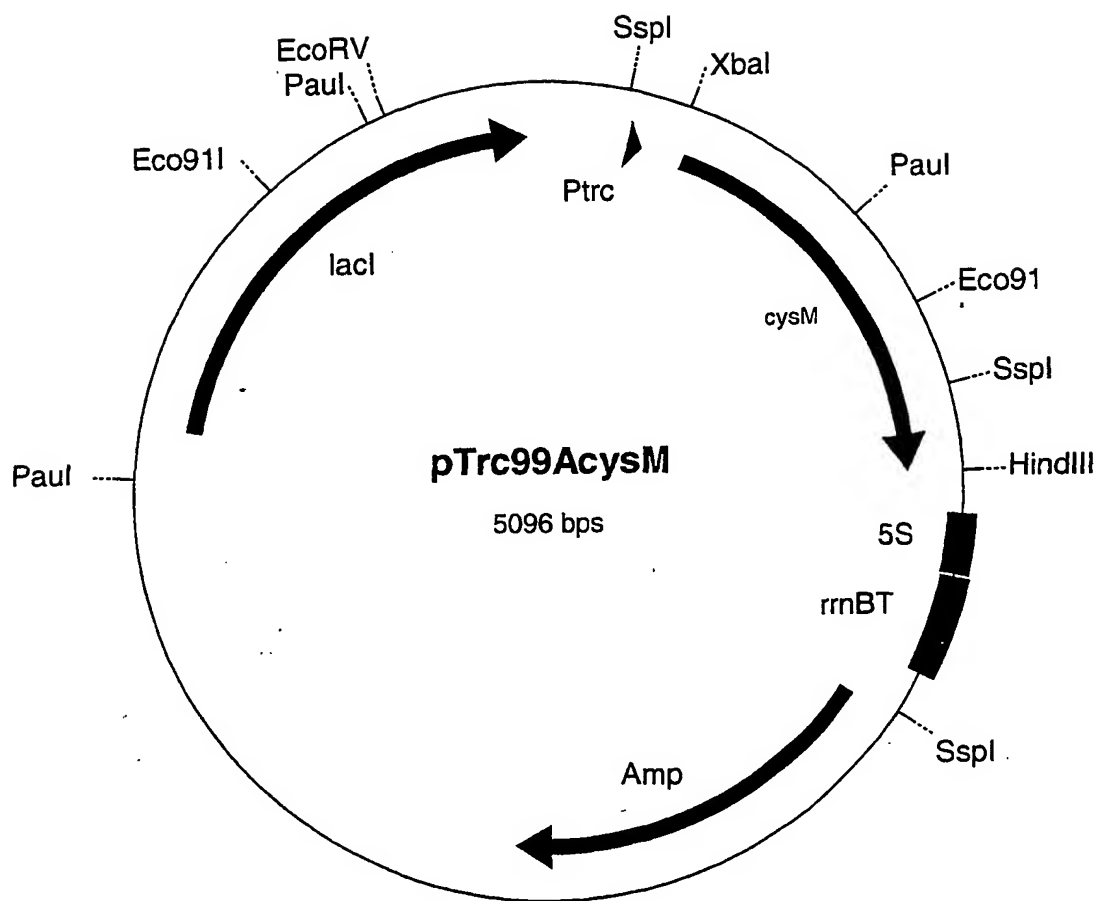


Figure 4:

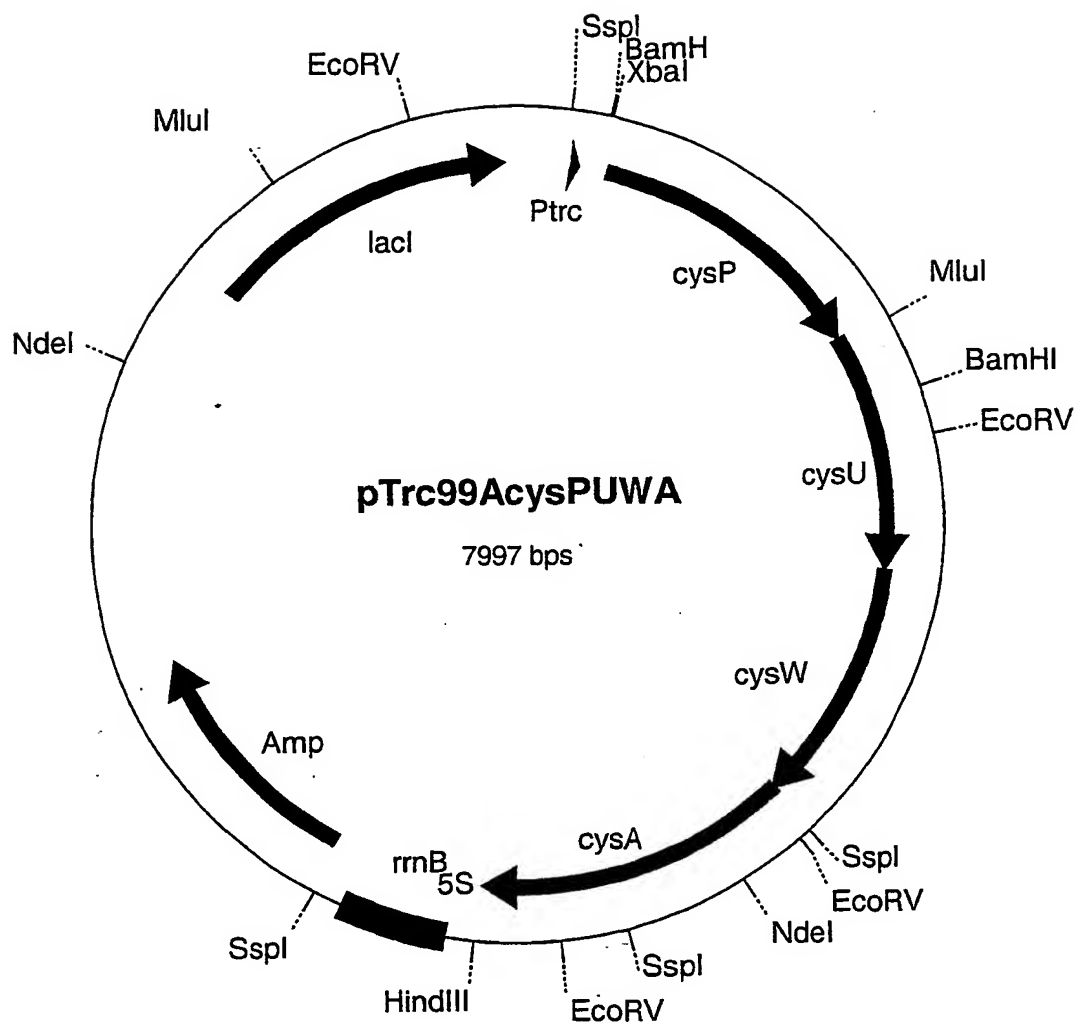


Figure 5:

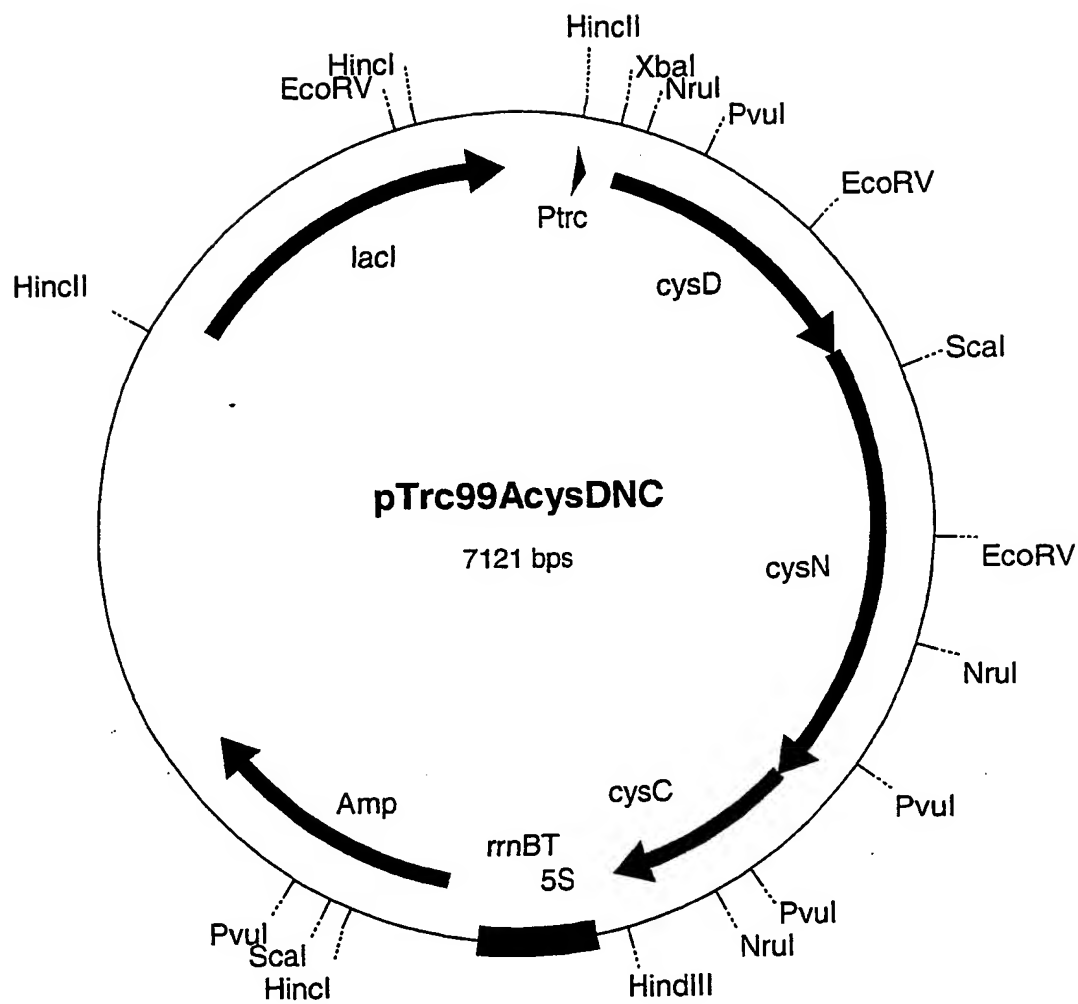


Figure 6:

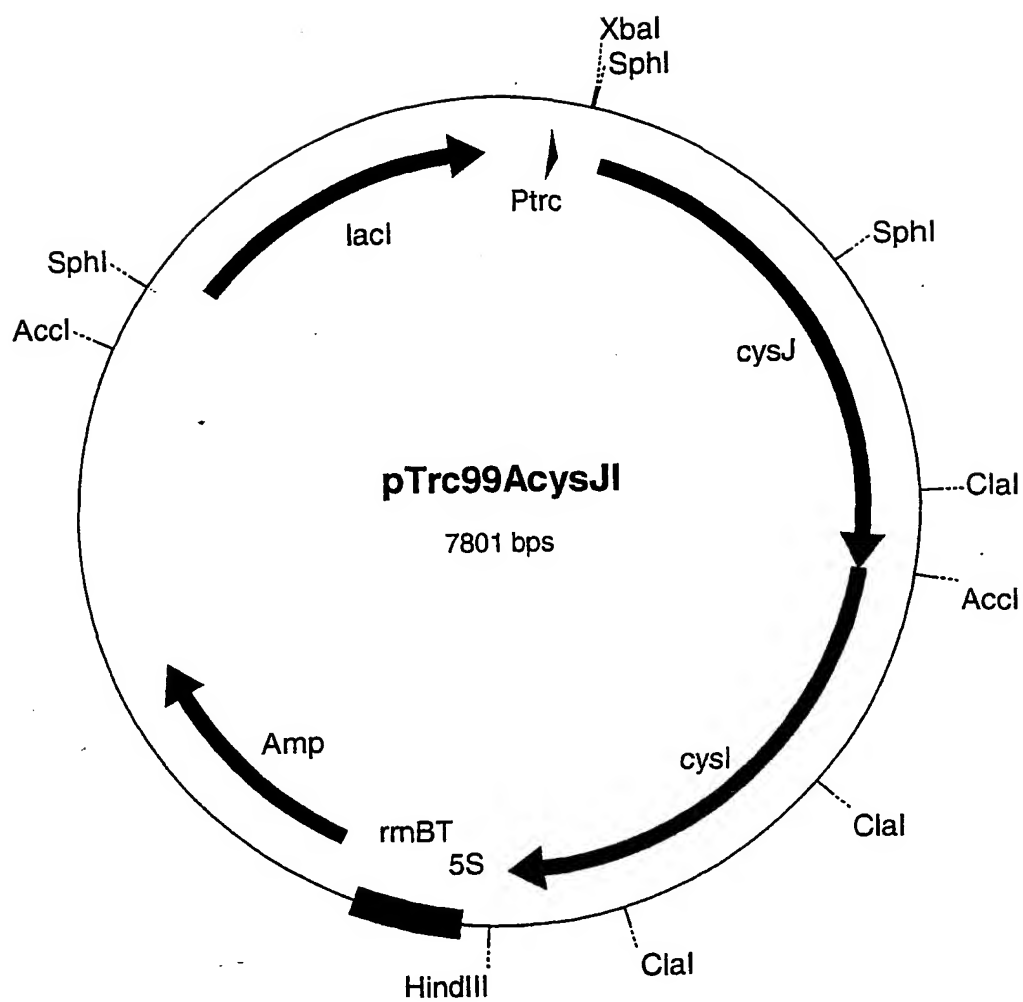
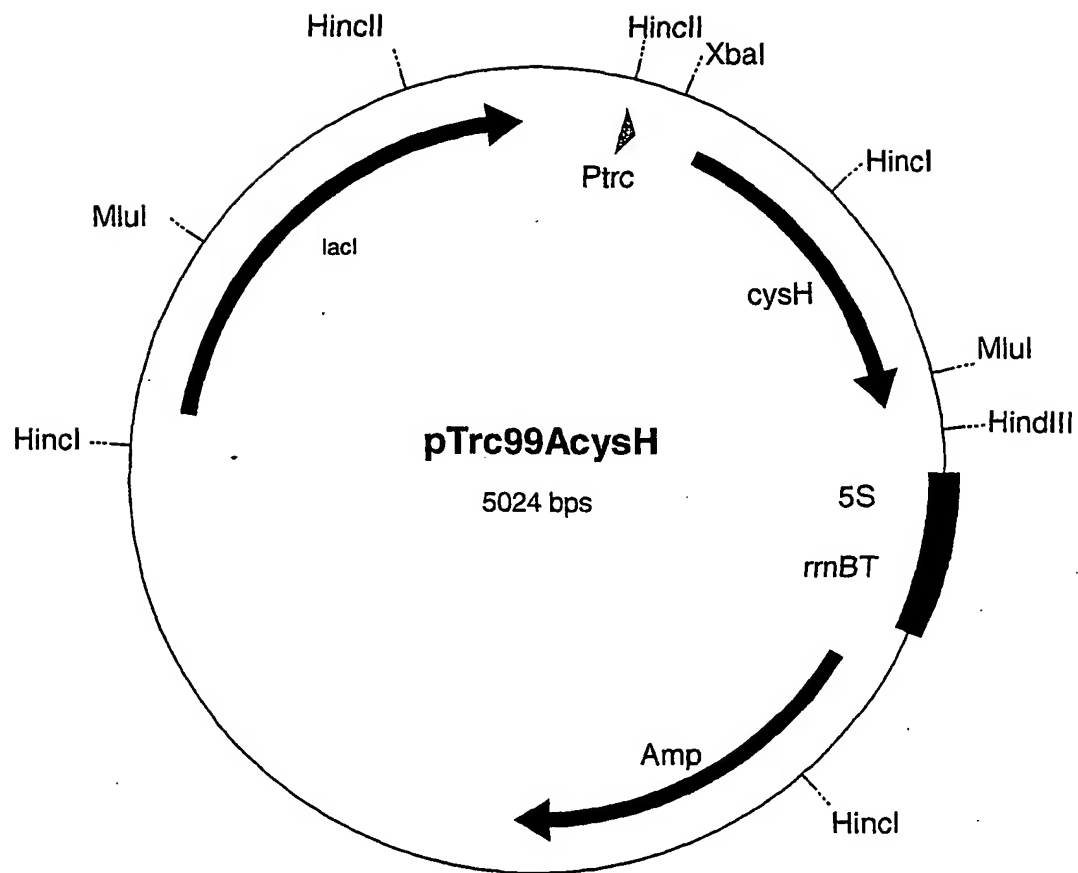


Figure 7:







<210> 5  
 <211> 28  
 <212> DNA  
 5 <213> artificial sequence  
  
 <220>  
 <221> Primer  
 <222> (1)..(28)  
 10 <223> cysM1  
  
 <400> 5  
 cgcacgagtc tagaccacgt taggatag 28  
  
 15 <210> 6  
 <211> 25  
 <212> DNA  
 <213> artificial sequence  
  
 20 <220>  
 <221> Primer  
 <222> (1)..(25)  
 <223> cysM2  
  
 25 <400> 6  
 catcagtcgc cgaagctttt aatcc 25  
  
 <210> 7  
 <211> 30  
 30 <212> DNA  
 <213> artificial sequence  
  
 <220>  
 <221> Primer  
 35 <222> (1)..(30)  
 <223> cysPUWA1  
  
 <400> 7  
 gtctctagat aaataagggt gcgcaatggc 30  
 40  
 <210> 8  
 <211> 25  
 <212> DNA  
 <213> artificial sequence  
 45  
 <220>  
 <221> Primer  
 <222> (1)..(25)  
 <223> cysPUWA2  
 50  
 <400> 8  
 ccgggcgttt aagcttcact caacc 25  
  
 <210> 9  
 <211> 30  
 <212> DNA  
 <213> artificial sequence  
  
 <220>  
 <221> Primer  
 60 <222> (1)..(30)  
 <223> cysDNC1

<210> 10  
 <211> 25  
 <212> DNA  
 5 <213> artificial sequence  
  
 <220>  
 <221> Primer  
 <222> (1)..(25)  
 10 <223> cysDNC2  
  
 <400> 10  
 catggaaagc ttgtggtgtc tcagg 25  
  
 15 <210> 11  
 <211> 22  
 <212> DNA  
 <213> artificial sequence  
  
 20 <220>  
 <221> Primer  
 <222> (1)..(22)  
 <223> cysJI1  
  
 25 <400> 11  
 ctggaacata acgacgcatg ac 22  
  
 <210> 12  
 <211> 21  
 30 <212> DNA  
 <213> artificial sequence  
  
 <220>  
 <221> cysJI2  
 35 <222> (1)..(21)  
 <223> cysJI2  
  
 <400> 12  
 gaccgggctg atgggtaatc c 21  
 40  
 <210> 13  
 <211> 22  
 <212> DNA  
 <213> artificial sequence  
 45  
 <220>  
 <221> Primer  
 <222> (1)..(22)  
 <223> cysH1  
 50  
 <400> 13  
 ggcaaacagt gaggaatcta tg 22  
  
 <210> 14  
 <211> 21  
 55 <212> DNA  
 <213> artificial sequence  
  
 <220>  
 <221> Primer  
 <222> (1)..(21)  
 60 <223> cysH2

cysH2  
 cysH2

21

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 January 2003 (23.01.2003)

PCT

(10) International Publication Number  
**WO 03/006666 A3**

(51) International Patent Classification<sup>7</sup>: C12P 13/06,  
13/08, 13/10, 13/12, 13/14, 13/20, 13/22, 13/24

(21) International Application Number: PCT/EP02/06187

(22) International Filing Date: 6 June 2002 (06.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
101 33 667.5 11 July 2001 (11.07.2001) DE  
60/305,144 16 July 2001 (16.07.2001) US

(71) Applicant (for all designated States except US): DE-  
GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): SIEBELT, Nicole  
[DE/DE]; Am Sennebach 28, 33397 Rietberg (DE).  
WIDAWKA, Petra [DE/DE]; Laerstrasse 12, 33615  
Bielefeld (DE). FARWICK, Mike [DE/DE]; Gus-  
tav-Adolf-Strasse 11, 33615 Bielefeld (DE).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,

CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,  
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,  
VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,  
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,  
NE, SN, TD, TG).

Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv)) for US only

Published:

— with international search report

(88) Date of publication of the international search report:  
13 November 2003

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which at least one or more of the genes of cysteine biosynthesis chosen from the group consisting of cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp, or nucleotide sequences thereof, is/are enhanced, in particular over-expressed, b) concentration of the desired L-amino acid in the culture supernatant and/or by isolation of the desired L-amino acid.



WO 03/006666 A3

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/06187

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>					
IPC 7	C12P13/06 C12P13/20	C12P13/08 C12P13/22	C12P13/10 C12P13/24	C12P13/12	C12P13/14
According to International Patent Classification (IPC) or to both national classification and IPC					
<b>B. FIELDS SEARCHED</b>					
Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12P					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, BIOSIS					
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>					
Category *	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
A	PEAKMAN T ET AL: "NUCLEOTIDE SEQUENCE, ORGANISATION AND STUCTURAL ANALYSIS OF THE PRODUCTS OF GENES IN THE NIRB - CYSG REGION OF THE ESCHERICHIA COLI K-12 CHROMOSOME" EUROPEAN JOURNAL OF BIOCHEMISTRY, BERLIN, DE, vol. 191, no. 2, July 1990 (1990-07), pages 315-323, XP001030905 ISSN: 0014-2956 cited in the application the whole document --- -/--				1-7
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.					
<b>* Special categories of cited documents :</b>					
"A" document defining the general state of the art which is not considered to be of particular relevance			"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention		
"E" earlier document but published on or after the international filing date			"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone		
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)			"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.		
"O" document referring to an oral disclosure, use, exhibition or other means			"Z" document member of the same patent family		
"P" document published prior to the international filing date but later than the priority date claimed					
Date of the actual completion of the international search 13 March 2003			Date of mailing of the international search report 03. 06. 2003		
European Patent Office, P.O. Box 1, 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl Fax: (+31-70) 340-3016			Kools, P		

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/06187

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WARREN M J ET AL: "Gene dissection demonstrates that the Escherichia coli cysG gene encodes a multifunctional protein."  THE BIOCHEMICAL JOURNAL. ENGLAND, vol. 302 ( Pt 3),  15 September 1994 (1994-09-15), pages 837-844, XP008014987  ISSN: 0264-6021  the whole document</p> <p>---</p>	1-7
A	<p>WOODCOCK S C ET AL: "Effect of mutations in the transmethylase and dehydrogenase/chelataase domains of sirohaem synthase (CysG) on sirohaem and cobalamin biosynthesis."  THE BIOCHEMICAL JOURNAL. ENGLAND, vol. 330 ( Pt 1),  15 February 1998 (1998-02-15), pages 121-129, XP002234624  ISSN: 0264-6021  the whole document</p> <p>---</p>	1-7
A	<p>DE 199 49 579 C (CONSORTIUM ELEKTROCHEM IND) 16 November 2000 (2000-11-16)  the whole document</p> <p>---</p>	1-7
A	<p>DE 195 39 952 A (CONSORTIUM ELEKTROCHEM IND) 30 April 1997 (1997-04-30)  the whole document</p> <p>---</p>	1-7
A	<p>OKAMOTO K ET AL: "HYPERPRODUCTION OF L-THREONINE BY AN ESCHERICHIA COLI MUTANT WITH IMPAIRED L-THREONINE UPTAKE"  BIOSCIENCE BIOTECHNOLOGY BIOCHEMISTRY, JAPAN SOC. FOR BIOSCIENCE, BIOTECHNOLOGY AND AGROCHEM. TOKYO, JP,  vol. 61, no. 11, November 1997 (1997-11), pages 1877-1882, XP001018682  ISSN: 0916-8451  the whole document</p> <p>-----</p>	1-7

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 02/06187

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7 partial

Patent Office

International Searching Authority

International Searching Authority

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-7 partial

Processes for the preparation of L-amino acids, which comprises fermentation of microorganism of the Enterobacteriaceae family which produce the desired L-amino acid in which the cysG gene, or the polynucleotide which code for this, is enhanced, or wherein the regulatory and/or catalytic properties of said gene are improved. Said process wherein at least one other gene selected from the group cysB, cysZ(K), cysM, cysA, cysW, cysU, cysP(J), cysD, cysN, cysC, cysI, cysH, cysE, or sbp is enhanced. A process wherein further genes of the biosynthesis pathway for the desired amino acid are additionally enhanced. Process wherein microorganisms are employed in which, in addition to the enhanced cysG gene, the metabolic pathways which reduce the formation of the desired amino acid are at least partly eliminated. Process wherein, in addition to the enhanced cysG gene, one or more additional amplified genes are selected from the group: thrABC, pyc, pps, ppc, pntA, pntB, rhtB, mgo, rhtC, thrE, gdhA, hns, pgm, fba, ptsH, ptsi, crr, ptsG, lrp, mopB, ahpC and/or ahpF. Process wherein in addition to the enhanced cysG gene, one or more attenuated genes are selected from the group: tdh, mdh, yjfa, yjfp, pckA, poxB, aceA, dgsA, fruR, and/or rpoS.

2. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysB gene.

3. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysZ (identical to the cysK) gene.

4. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysM gene.

5. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysA gene.

6. Claims: 1-7 partial

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

See subject 1, replacing in the text the cysG for the cysW gene.

7. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysU gene.

8. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysP (identical to the cysJ) gene.

9. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysD gene.

10. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysN gene.

11. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysC gene.

12. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysI gene.

13. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysH gene.

14. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the cysE gene.

15. Claims: 1-7 partial

See subject 1, replacing in the text the cysG for the sop



FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

gene.